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Detection of the host immune response to *Burkholderia mallei* heat-shock proteins GroEL and DnaK in a glanders patient and infected mice☆

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Abstract

We examined, by enzyme-linked immunosorbent assay and Western blot analysis, the host immune response to 2 heat-shock proteins (hsps) in a patient and mice previously infected with *Burkholderia mallei*. The patient was the first reported human glanders case in 50 years in the United States. The expression of the *groEL* and *dnaK* operons appeared to be dependent upon a σ^{32} RNA polymerase as suggested by conserved heat-shock promoter sequences, and the *groESL* operon may be negatively regulated by a controlling invert repeat of chaperone expression (CIRCE) site. In the antisera, the GroEL protein was found to be more immunoreactive than the DnaK protein in both a human patient and mice previously infected with *B. mallei*. Examination of the supernatant of a growing culture of *B. mallei* showed that more GroEL protein than DnaK protein was released from the cell. This may occur similarly within an infected host causing an elevated host immune response to the *B. mallei* hsps.
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Keywords: *Burkholderia mallei*; Heat shock proteins; Host response

1. Introduction

Heat-shock proteins (hsps) are one of the most highly conserved proteins in nature and play a critical role as molecular chaperones for newly synthesized proteins in the cell (Zugel and Kaufmann, 1999). In bacteria that are stressed, such as by changes in environmental conditions, the synthesis of these proteins in the cell is up-regulated. They participate in proper folding and assembly of selected polypeptides as they come off the ribosome, play a role in transporting proteins to different locations in the cell, and play a role in the degradation of aggregated or misfolded proteins in the cell. In addition, bacterial hsps readily elicit an immune response in the host for several reasons (van Eden et al., 2005): 1) the high conservation between hsps of bacterial cells ensures that the host is primed to respond to the presence of common epitopes on the hsps; 2) under the stress of establishing an infection, the bacteria up-regulates the synthesis of hsps to make them more readily available to interact with the host's immune system; and 3) hsps associated with newly synthesized or misfolded microbial proteins can be taken up by professional antigen-presenting cells, which process both proteins through the major histocompatibility complex class I and II pathways (Shinnick, 1991; Stewart and Young, 2004; van Eden et al., 2005).

Very little is known about the hsps from *Burkholderia mallei*, the causative agent of glanders. *B. mallei* is a Gram-negative bacillus, which causes glanders primarily in horses, mules, and donkeys. Oral infections or contact with an infected animal are the most common methods of acquiring glanders because the organism can be transmitted through droplets or saliva. In horses, glanders is often manifested as a slow progressive chronic disease, but in donkeys and mules, the disease often takes an acute course with death occurring within a week to 10 days (Acha and Szyfres, 1987; Domma, 1953). Involvement of

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14. ABSTRACT

We examined, by enzyme-linked immunosorbent assay and Western blot analysis, the host immune response to 2 heat-shock proteins (hsps) in a patient and mice previously infected with Burkholderia mallei. The patient was the first reported human glanders case in 50 years in the United States. The expression of the groEL and dnaK operons appeared to be dependent upon a sigma(32) RNA polymerase as suggested by conserved heat-shock promoter sequences, and the groESL operon may be negatively regulated by a controlling invert repeat of chaperone expression (CIRCE) site. In the antisera, the GroEL protein was found to be more immunoreactive than the DnaK protein in both a human patient and mice previously infected with B. mallei. Examination of the supernatant of a growing culture of B. mallei showed that more GroEL protein than DnaK protein was released from the cell. This may occur similarly within an infected host causing an elevated host immune response to the B. mallei hsps.

15. SUBJECT TERMS
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the lungs and upper respiratory tract is common and is manifested by a regional or diffuse pneumonia or pleuritis, and a nasal exudate, which is infectious. Cutaneous infections are manifested by enlarged and indurated (farcy) regional lymph nodes, which may rupture and suppurate. There is no effective treatment of glanders in the natural host, so animals diagnosed with glanders are isolated and destroyed. In humans, infection by *B. mallei* may be through the oral, nasal, ocular, or cutaneous routes. It was not known that *B. mallei* was highly infectious for humans until the organism was studied under laboratory conditions. It was noted that within the first year of study, half the workers became infected with glanders most likely through an aerosol route (Waag and DeShazer, 2004; Howe and Miller, 1947). In a more recent report, glanders was obtained possibly through a cutaneous route by an investigator, and once the organism was identified, a 6-month treatment with a combination of azithromycin and doxycycline was effective in resolving the infection (Srinivasan et al., 2001). Progression of the disease is similar to that seen in the natural hosts and may present as an acute localized or pulmonary form, which can lead to a fatal septicemic illness. A chronic lymphangitis and regional adenopathy may also be seen. Usually, in humans a combination of the symptoms is seen.

Our hypothesis is that the *B. mallei* GroEL (the *Escherichia coli* homologue for the large 60-kDa hsp) and DnaK (the *E. coli* homology for 70-kDa hsp) proteins, which are molecular chaperones that are from the *groESL* and *dnaK* operons, respectively, are immunogenic in a glanders patient, and because of their ability to induce a T-cell response in the host, they can potentially be used as part of a vaccine to prevent or ameliorate glanders infection in humans. In our studies reported here, we examined the host immune response of an infected patient and mice to the *B. mallei* hsps GroEL and DnaK proteins. The infected patient was the first reported human glanders case in 50 years in the United States (Srinivasan et al., 2001). The GroEL protein was found to be more immunoreactive than the DnaK protein in antisera from a patient and mice previously infected with *B. mallei*. More GroEL protein appeared to be released from the cells during growth than the DnaK protein, which may explain the host's differential immune response to these hsps from *B. mallei*.

2. Materials and methods

2.1. Antiserum, bacterial cells, and proteins

Antisera from the *B. mallei*-infected patient (Srinivasan et al., 2001) and healthy volunteer patients were obtained from the US Army Medical Research Institute of Infectious Diseases (USAMRIID, Fort Detrick, MD). Preexisting anonymous specimens were approved for purposes of this study by the USAMRIID Human Use Committee, which determined the specimens met conditions for exemption category 4 [45 CFR 46.101(b)(4)]. Mouse antisera were obtained from uninfected BALB/c mice or previously infected BALB/c mice, which had survived a low aerosol challenge of *B. mallei* ATCC 23344. *B. mallei* cells used for antibody studies were prepared as previously described (Amemiya et al., 2002). The *B. mallei* strain (pBH1-GFP) containing green fluorescent protein (GFP) was constructed as previously described (Stevens et al., 2005). Recombinant GFP was obtained from BioVision Research Products, Mountain View, CA, and chicken anti-GFP antibody was obtained from Upstate USA, Charlottesville, VA. The plague candidate vaccine F1-V was obtained from Dr. Brad Powell here at USAMRIID, and anthrax vaccine recombinant protective antigen (rPA) was obtained from Dr. Bruce Ivins (USAMRIID).

Research was conducted in compliance with the Animal Welfare Act and other federal statues and regulations relating to animals and experiments involving animals, and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, National Research Council, 1996. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

2.2. Cloning, overexpression, and purification of hsps

We began our cloning and sequencing of the *B. mallei groEL* and *dnaK* genes before they were completed and annotated by The Institute of Genomic Research (TIGR) at www.tigr.org/. The *B. mallei groEL* and *dnaK* genes were cloned by using polymerase chain reaction (PCR) primers with sequences obtained from the sequences of the genes from *Burkholderia pseudomallei* deposited in GenBank (AF287633 and AF016711, respectively) and *B. mallei* ATCC 23344 DNA. The *B. pseudomallei* DNA sequences (Holden et al., 2004) were used because of the close homology (99%) between the genomic sequences of *B. mallei* and *B. pseudomallei*. The sequences of the primers used for the *groEL* gene were as follows: sense, 5′-ATGGCAGCTAAAGACGTCG-3′; antisense, 5′-TTACATGTCCATGCCCAT-3′. The sequences of the *dnaK* gene primers were as follows: sense, 5′-ATGGGAAAGATCATCGGTATTGACC-3′; antisense, 5′-TCAGTCCTTCTTCACTTCCTGAAG-3′. After the PCR reaction, the respective products were purified from the agarose gel and cloned into the pCR T7/NT-TOPO vector (Invitrogen, Carlsbad, CA). The genes were sequenced and subsequently subcloned into a Multisite Gateway vector (Invitrogen), DNA sequence confirmed, and proteins overexpressed and purified by the Protein Expression and Purification Laboratory at the National Cancer Institute, Frederick, MD. The his6-MBP-tev-tag was cleaved from each protein before the final purification of the protein. The endotoxin levels (Cambrex Bio Science, Walkersville, MD) for the GroEL and DnaK preparations were 0.22 and 8 EU/mg, respectively.

2.2.1. Nucleotide sequence accession number

The nucleotide sequences of the *B. mallei groEL* and *dnaK* genes were deposited with GenBank under accession no. DQ061983 and DQ061984, respectively.

2.3. Genes and regulatory sequences of the B. mallei groESL and dnaK operons

DNA sequence homology between the *B. mallei*, *B. pseudomallei*, and *E. coli groEL* and *dnaK* genes and open reading frames (orfs) in the region of the *B. mallei groESL* and *dnaK* operons were determined with MacVector 7.0 software (Accelrys, Burlington, MA). The DNA sequence (contigs) in the region of the *B. mallei groEL* and *dnaK* genes were obtained from the TIGR web site (www.tigr.org).

2.4. Antibody assays

Antibody titers were determined by enzyme-linked immunosorbent assay (ELISA) as described previously (Amemiya et al., 2002). Briefly, 2-fold dilutions of antisera were made in triplicate in 96-well Immulon 2HB plates (Thermo Labsystems, Franklin, MA), which previously contained 50 μL of nonviable *B. mallei* cells (whole-cells) (10 μg/mL) or proteins (2 μg/mL) in 0.1 mol/L carbonate buffer, pH 9.5. Antibody class was determined with anti–Ig-horseradish peroxidase conjugate (Southern Biotechnology Associates, Birmingham, AL). The results are reported as the geometric mean of the reciprocal of the highest dilution giving an outer diameter of at least 0.1 at 450 nm on a Dynex MRX instrument (Dynex Technologies, Chantilly, VA), and the SE.

2.5. Western blot analysis

Proteins samples (0.25–0.5 μg) were analyzed on 10% tricine gels (Invitrogen) and visualized by staining the gel with Simply Blue Safestain (Invitrogen). For Western blot analysis, proteins were transferred to nitrocellulose membrane filters (Invitrogen), probed with antibodies, which were at the same dilution. Unless stated, antibodies from the human patient were diluted 1:1000 for Western blot studies, and antibodies from mice for the Western blot

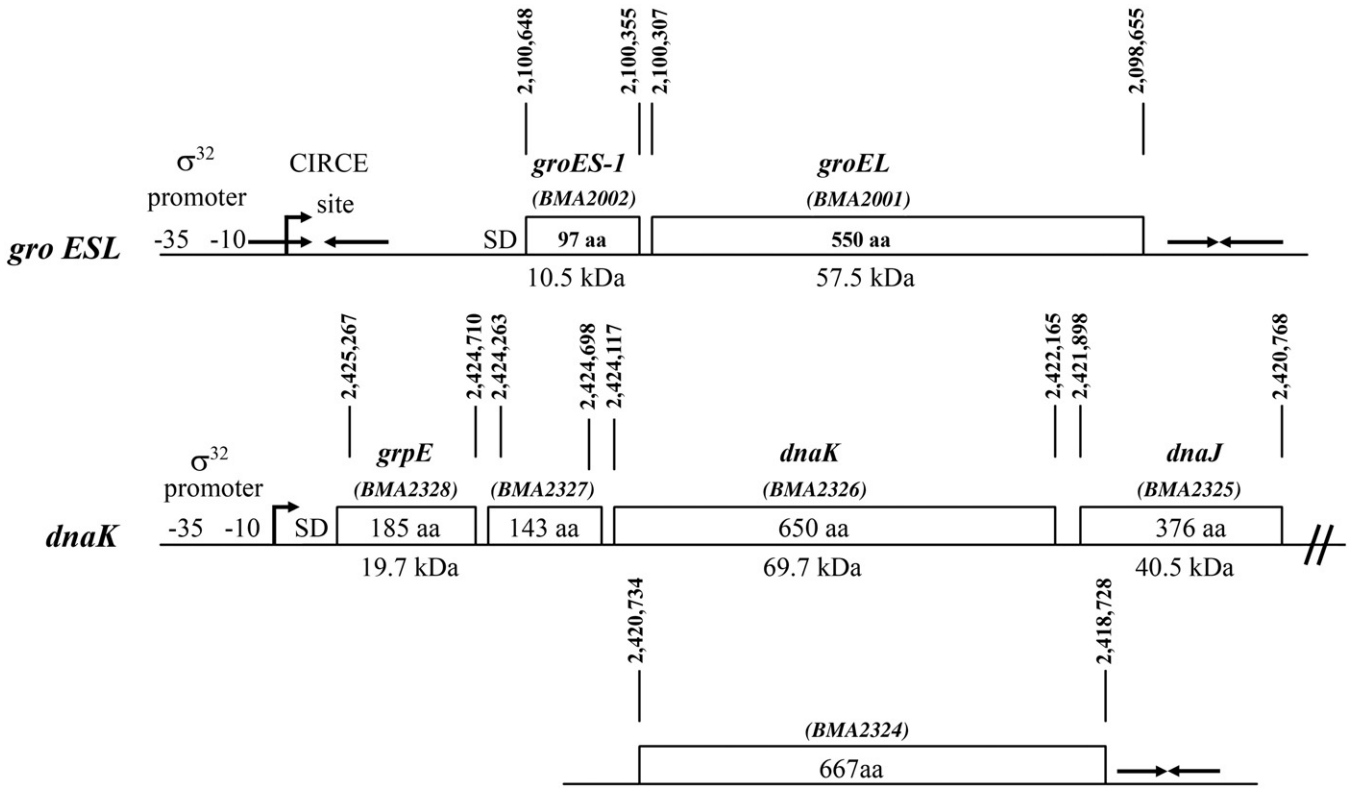

Fig. 1. Organization and putative promoter region of the *B. mallei* heat shock *groESL* and *dnaK* operons. Upper figure shows the position of the *groEL* gene within the *groESL* operon, and the lower figure shows the position of the *dnaK* gene within the *dnaK* operon. The relative position of the σ^{32} RNA polymerase promoter consensus sequence is shown on the left of both operons. The bent arrow shows the direction of transcription, and the IR in the promoter region of the *groESL* operon represents the relative location of a CIRCE consensus sequence (Zuber and Schumann, 1994). SD represents a Shine–Dalgarno ribosome-binding sequence. At the 3′ end of each operon is shown the presence of a large IR sequence. The vertical numbers above each orf are the position in the *B. mallei* chromosome ATCC 23344 reported by TIGR. The annotations above each orfs in parenthesis are the locus name given by TIGR, and the name of the protein is shown above the locus unless the gene was unknown or questionable. The putative molecular mass of the protein encoded by the orf is shown below.

studies were diluted 1:2000. The protein–antibody complexes were visualized using a SuperSignal West Pico Chemiluminescent Substrate kit (Pierce Biotechnology, Rockford, IL) with CL-X Posure film (Pierce). Western blots were exposed for the same length of time for comparison, except for the control blots, which were exposed for longer periods.

2.6. Growth of B. mallei cultures

Previously frozen stocks of *B. mallei* (ATCC 23344) or an isogenic strain containing a plasmid (pBHR1-GFP) that expresses GFP were inoculated into 3 mL of LB broth (Lennox L broth; Sigma, St. Louis, MO) containing 4% glycerol (Luria broth with glycerol) or 3 mL of LBG with 5 μg/mL of kanamycin, respectively. The cultures were incubated at 37 °C with shaking at 250 rpm for 16 h. The next day, 300 μL of the overnight cultures were used to inoculate 3 mL of the appropriate fresh medium, and cultures were incubated as described above. At the desired periods, 1-mL aliquots of the growing cells were removed and centrifuged to pellet the cells. The cell-free supernatant was filtered through a 0.45-μm Millex-HA filter (Millipore, Bedford, MA), and 100 μL of the filtered supernatant was plated out onto LBG agar to check for growth.

2.7. Statistical analysis

Log_{10} transformations were applied to titer values before analysis. Analysis of variance (ANOVA) with post hoc Dunnett's tests was used to compare titers at each time point to pooled normal sera. ANOVA with post hoc Dunnett's tests was also used to compare titers of exposed mice to the titers of unexposed control mice. Analyses were conducted using SAS Version 9.1 (SAS Institute 2004, SAS OnlineDoc® 9.1.3; SAS Institute, Cary, NC).

3. Results

3.1. Nucleotide sequence of the B. mallei groEL and dnaK genes, and the organization of their operons

To examine the host's immune response to the *B. mallei* GroEL and DnaK hsps, we cloned and sequenced the 2 hsps from *B. mallei* ATCC 23344. We started this study before the sequences of the hsp genes from *B. mallei* were available and annotated by TIGR and, therefore, used the hsp sequences from *B. pseudomallei* to design the PCR primers for cloning. The *groEL* gene was 1653 base pairs (bp) long and was 99% identical with that of the *groEL* gene reported for *B. pseudomallei* (Holden et al., 2004; Woo et al., 2001). The *B. mallei* gene was 12 bp longer than the *B. pseudomallei* gene, which was due to 2 identical repeats (nt 1621–1632 and 1633–1644) present at the 3′ end of the *B. mallei* gene. There was another pair of different 12-bp repeats immediately upstream (nt 1591–1602 and 1603–1614) and were present in both *B. mallei* and *B. pseudomallei groEL* genes. Our nt sequence of *groEL* was the same as that eventually reported by TIGR (designated *BMA2001*). Fig. 1A shows the organization of the genes in the *groESL* operon of *B. mallei* as deduced from the DNA sequence by TIGR. There is only one copy of the *groEL* gene on the large chromosome (as opposed to the small chromosome), but TIGR reported that there are 2 copies of the *groES* genes (which encodes a 10-kDa small regulatory chaperone that forms a heptamer that complexes with GroEL), both on the large chromosome. The *groES*-1 gene (*BMA2002*), which is located within the *groESL* operon, was only 61% identical on the nucleotide level and 54% identical on the amino acid (aa) level with the second *groES*-2 gene (*BMA2431*) in another region of the large chromosome.

We found a putative recognition site for the heat-shock σ^{32} RNA polymerase 106-nt upstream from the start of the

Table 1
IgG antibody titers against various antigens in a glanders patient and healthy volunteers

Patient sample	Antibody titer[a]			
	Antigens			
	B. mallei GroEL	*B. mallei* DnaK	*B. mallei* Whole Cell	*B. anthracis* (protective antigen)
Glanders				
Preinfection	100 794 (1.5)[b, ***]	50 797 (1.5)***	20 160 (1.5)	320 000 (1.0)***
2 months post	1 015 937 (1.5)***	80 000 (1.0)***	512 000 (1.0)***	2 560 000 (1.0)***
3 months post	1 280 000 (1.0)***	1 612 699 (1.5)***	320 000 (2.0)***	5 120 000 (1.0)***
5 months post	508 000 (1.5)***	160 000 (1.0)***	201 600 (1.5)***	5 120 000 (1.0)***
8 months post	403 175 (1.5)***	80 000 (1.0)***	512 000 (1.0)***	403 175 (1.5)***
9 months post	80 000 (1.0)***	100 794 (1.0)***	512 000 (1.0)***	2 560 000 (1.0)***
13 months post	201 587 (1.5)***	126 992 (1.0)***	203 200 (1.5)***	10 240 000 (1.0)***
Healthy volunteers				
Serum 1	12 800 (1.0)	8063 (1.5)	16 127 (1.5)	<50[c]
Serum 2	16 127 (1.5)	12 800 (1.0)	32 000 (1.0)	<50

[a] Antibody titers were determined in triplicate and reported as the geometric mean ± SD.
[b] When compared with infected or vaccinated versus uninfected or unvaccinated.
[c] Antibody titers of 50 or less are considered to be negative.
*** $P < 0.0001$.

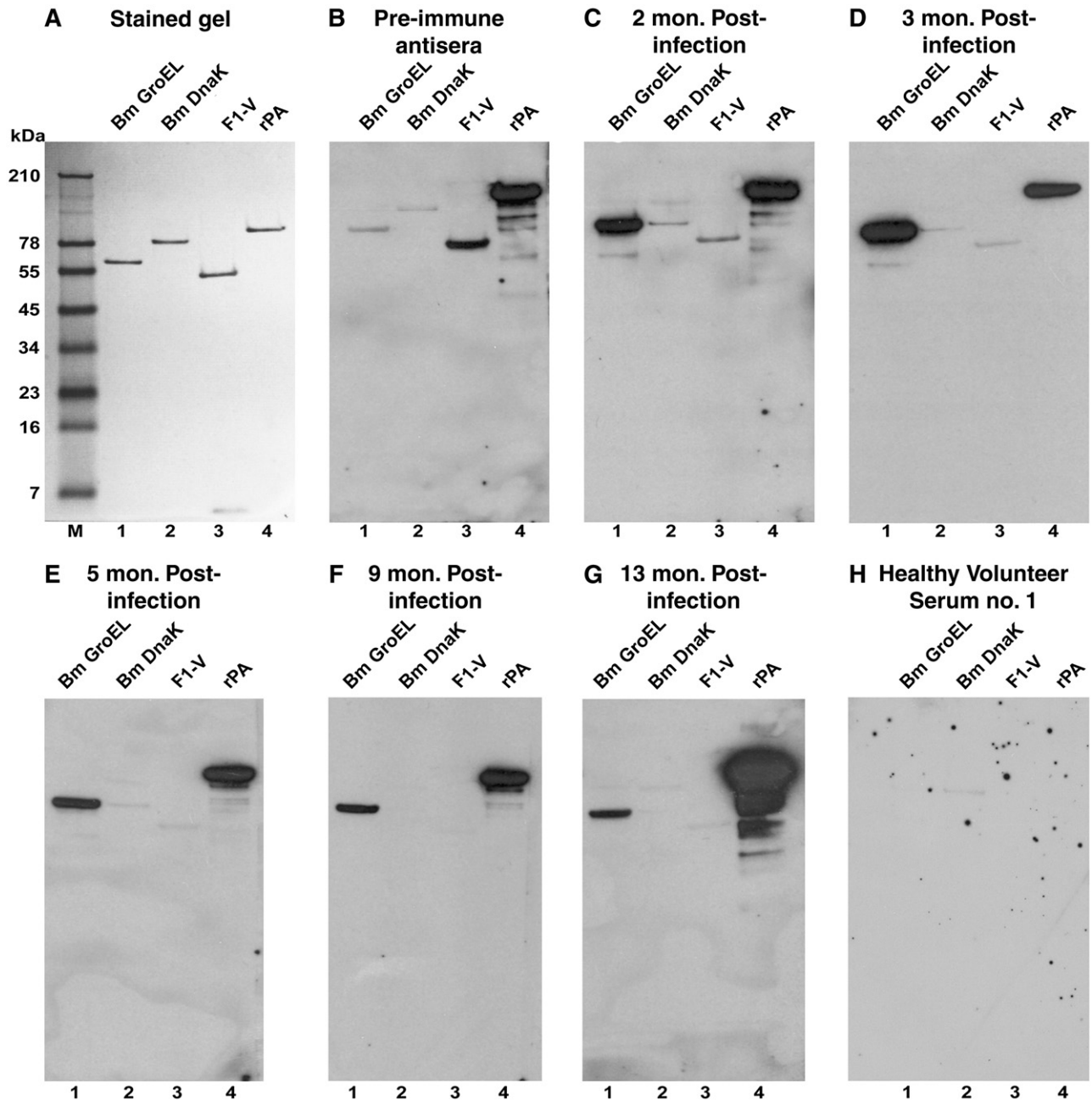

Fig. 2. Western blot analysis of anti-GroEL and anti-DnaK immune reactivity in the serum of a patient infected with *B. mallei*. All panels contain the following antigens: lane 1, *B. mallei* (Bm) GroEL; lane 2, Bm DnaK; lane 3, *Y. pestis* plague control vaccine F1-V; lane 4, *B. anthracis* control rPA. Panel A represents a stained gel with size markers (M). Panels B through G are Western blots probed with the patients antiserum obtained at different times after infection: preimmune, 2 months postinfection, 3 months postinfection, 5 months postinfection, 9 months postinfection, 13 months postinfection. Panel H represents a Western blot probed with a healthy volunteer (no. 1 patient in Table 1) antiserum. All antisera were used at a 1 of 1000 dilution and blots exposed for 10 min, except the control, which was exposed for 20 min.

groES gene (Fig. 1, top) and also a consensus controlling inverted repeat of chaperone expression (CIRCE) site beginning 77 nt upstream from the gro*ES* gene (Zuber and Schumann, 1994) (Fig. 1, top). This is the binding site for the negative regulatory protein called the heat regulation at CIRCE or HrcA (Roberts et al., 1996). Identical regulatory sequences were also present the same distance upstream from the *groES* gene in *B. pseudomallei* (data not shown). The *hrcA* gene (*BMA2331*) appeared to be in another operon located just upstream from the *dnaK* operon. Also, 49 bp downstream from the 3′ end of the *groEL* gene, we found a 22-nt-long invert repeat (IR) sequence with one mismatched base, which may be the transcription termination signal for the operon.

The *dnaK* gene from *B. mallei* was 1953 bp long and appeared to be within an operon with 4 other genes or orfs

Table 2
IgG immune response to *B. mallei* hsps in mice exposed to a low aerosol dose of *B. mallei*

Sample no.	Antibody titer[a]		
	Antigens		
	B. mallei GroEL	*B. mallei* DnaK	*B. mallei* Whole Cell
Control	79 (1.5)	1270 (1.5)	100 (2.0)
Mouse 1	2 560 000 (1.0)[b, ***]	256 000 (1.0)***	1 280 000 (1.0)***
Mouse 2	2 560 000 (1.0)***	101 594 (1.5)***	640 000 (1.0)***
Mouse 3	253 984 (1.5)***	25 398 (1.5)***	253 984 (1.5)***
Mouse 4	160 000 (1.0)***	32 000 (1.0)***	160 000 (1.0)***

[a] Antibody titer preformed in triplicate and reported as the geometric mean ± SE.
[b] When compare with exposed mice versus unexposed mice.
*** $P < 0.0001$.

(Fig. 1, bottom). At the 5′ end of the operon was the *grpE* gene (encodes for the cochaperone GroP-like gene E protein, which is a nucleotide exchange factor that complexes with DnaK) (*BMA2328*) followed by a 143-aa orf (*BMA2327*, hypothetical protein). The next orf contained the *dnaK* gene (*BMA2326*), which was followed by the *dnaJ* gene (encodes a chaperone that is involved with protein folding and accelerates hydrolysis of adenosine triphosphate with DnaK) (*BMA2325*). The last apparent orf of the operon consisted of 667 aa, which was annotated as a chorismate binding enzyme (*BMA2324*). Ninety-five nt 5′ to the *grpE* gene were sequences that appeared to be the recognition site for the heat-shock σ^{32} RNA polymerase (Fig. 1, bottom). The results suggest that the expression of both the *B. mallei groEL* and *dnaK* operons are under the positive control of a heat-shock σ^{32} RNA polymerase, and that the expression of the *groESL* operon may also be under the control of a negative regulatory protein (HrcA).

3.2. Antibodies to the B. mallei GroEL and DnaK proteins can be detected by ELISA in antisera from a B. mallei-infected patient

To determine if the *B. mallei* GroEL and DnaK proteins induced an immune response in a patient infected with *B. mallei* (Srinivasan et al., 2001), we used the hsp proteins as capture antigens in an ELISA. Table 1 shows that the IgG response to the *B. mallei* GroEL and DnaK proteins increased from the preinfection period to the third month after the infection was diagnosed and decreased thereafter to 13 months postinfection. Overall, the antibody response to GroEL was generally higher than the immune response to DnaK. The antibody response to *B. mallei* whole cells likewise increased from the preinfection period to the 2 to 3 months postinfection and stayed at that level to 13 months postinfection. When the IgG titers of the patient to the *B. mallei* antigens were compared with the titers of healthy volunteers, all were significantly higher ($P < 0.0001$), except the preinfection titer against the whole-cell antigen. Serum from the healthy volunteers reacted slightly to these same antigens, although generally not as much as found in the preinfection sample from the glanders patient. We also examined the antibody response to *Bacillus anthracis* rPA as a non-*B. mallei* control antigen and found that there was a good antibody response to this protein during the glanders infection. We subsequently learned that the glanders patient had received the *B. anthracis* vaccine (anthrax vaccine adsorbed) for anthrax and, during the convalescent period, had received an annual booster, as evidenced by the increase in IgG titer in the 9- and 13-month postinfection samples. The sera from healthy volunteers were negative for the anthrax vaccine. An IgM immune response was also detected against the hsps (data not shown). In conclusion, both GroEL and DnaK proteins were immunogenic, as evaluated by ELISA, when tested with serum from a glanders patient, although the GroEL protein was generally more immunoreactive than the DnaK protein.

3.3. The GroEL protein is more immunoreactive than the DnaK protein by Western blot analysis in a glanders patient

Because it was previously reported that GroEL was highly antigenic in melioidosis patients infected with the closely related *B. pseudomallei* by Western blot analysis (Woo et al., 2001), we examined the immune response to GroEL and DnaK in antisera from a glanders patient by Western blot analysis. Fig. 2B shows that there was a weak immune response to both GroEL and DnaK from the patient's preimmune antisera. We also examined the immune reactivity to the *Yersinia pestis* plague vaccine F1-V and the *B. anthracis* anthrax vaccine rPA as controls, and found that they were both immunoreactive in the preimmune antisera. We used 2 non-*B. mallei* control proteins because we wanted at least one negative control protein in the Western blot analysis. However, we subsequently learned that the patient had previously been vaccinated with the United States Pharmacopeia whole-cell plague vaccine as well as with the anthrax vaccine (Table 1). At 2 months postinfection (Fig. 2C), we detected reactivity to the *B. mallei* GroEL, the plague F1-V, and the anthrax recombinant (r) PA proteins. At the same time, the *B. mallei* DnaK protein was not as immunoreactive by Western blot analysis. The light band migrating at the same size as the GroEL protein in Fig. 2C, lane 2 (and Fig. 2D, lane 2), could be a spillover from the adjacent GroEL sample. At 3 months postinfection (Fig. 2D), the reactivity to the GroEL protein was strong, whereas the reactivity to the DnaK protein was not detected. At the same time, the reactivity to the plague F1-V protein was very weak, whereas the reactivity to rPA was still high. In the 5 to 13 months postinfection antisera (Fig. 2E–G), we could detect reactivity to the *B. mallei* GroEL protein but not to DnaK. Under the same conditions, we could not detect reactivity to the plague F1-V vaccine, but the reactivity to rPA was very high, with the highest reactivity to rPA seen at 13 months postinfection. The patient had a booster vaccination to anthrax several months before. The healthy volunteer serum (no. 1) had no reactivity to any of the antigens tested by

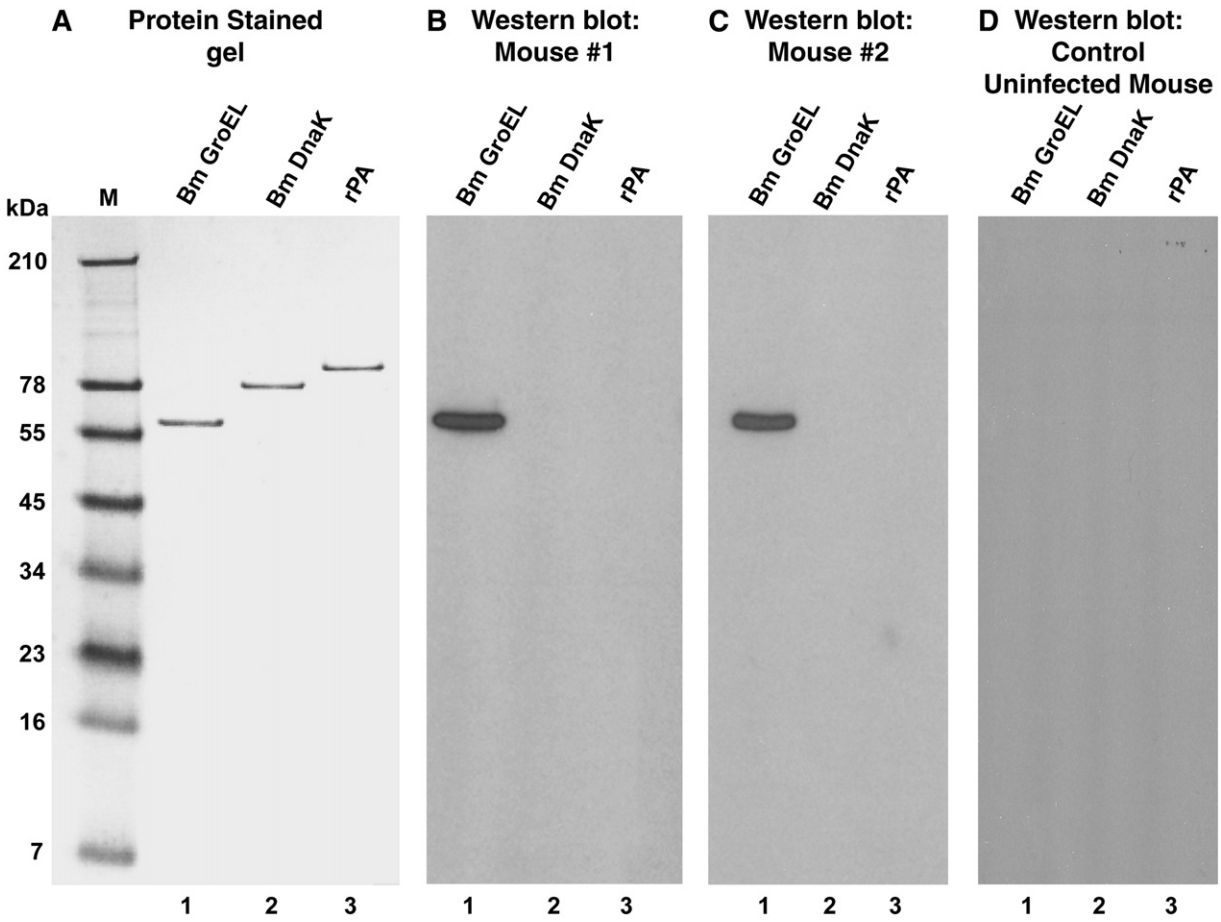

Fig. 3. Western blot analysis of anti-GroEL and anti-DnaK immune activity in antisera from BALB/c mice previously infected with *B. mallei*. All panels contained the following antigens: lane 1, *B. mallei* (Bm) GroEL; lane 2, Bm DnaK; lane 3, *B. anthracis* control rPA. Panel A shows a stained gel with the size markers (M, and the antigens). Panels B and C were Western blots probed with antisera from previously *B. mallei* infected mice. Panel D was a Western blot probed with uninfected mouse antiserum. All Western blots were probed with the respective mouse antisera diluted 1 of 2000 and exposed for the same period.

Western blot analysis (Fig. 2H), although the blot was exposed for twice the time as the blots of the glanders patient. Weak reactivity to DnaK and F1-V could be seen on higher exposures of the Western blot (data not shown). In summary, the *B. mallei* GroEL protein was more immunoreactive than the DnaK protein in the antisera from the glanders patient, when examined by Western blot analysis.

3.4. B. mallei GroEL is more immunogenic than DnaK in mice infected with B. mallei

Because GroEL and DnaK were immunogenic in a glanders-infected patient, we wanted to evaluate the immunogenicity of the proteins in mice that were infected with *B. mallei*. We examined the antisera of 4 mice exposed to *B. mallei* by ELISA against the *B. mallei* GroEL, DnaK, and *B. mallei* WC as the capture antigens (Table 2). In all cases, the IgG titer to GroEL was much higher (10-fold higher in most cases) than the IgG titer to DnaK (data not shown). The titer to both of these proteins was significantly ($P < 0.0001$) higher than the titers from unexposed mice. There was also a significant IgG and IgM (data not shown) immune response to *B. mallei* whole cells in the same mice.

When we evaluated the murine immune response of 2 of the *B. mallei*-exposed mice to GroEL and DnaK proteins by Western blot analysis, we could only detect an immune response to the GroEL protein (Fig. 3). We could not detect antibodies to DnaK by Western blot even at higher exposures (data not shown). The antiserum from the uninfected control mouse was negative to the 2 hsps and the control antigen *B. anthracis* rPA protein. Similar to what we observed in the human glanders case, the GroEL protein was more immunoreactive than the DnaK protein in glanders-infected mice, as evaluated by ELISA and Western blot analysis.

3.5. The GroEL protein is more immunoreactive than the DnaK protein in hosts because more GroEL appears to be released from B. mallei during growth

We wanted to determine why the GroEL protein was more immunoreactive in hosts infected with *B. mallei*. It could be that the GroEL protein was somehow exposed to the immune system of the host whereas the DnaK protein was not. However, murine fluorescent-labeled antibody to GroEL or DnaK proteins was not significantly associated with the surface of the cell. There were a few cells (~2%) that were

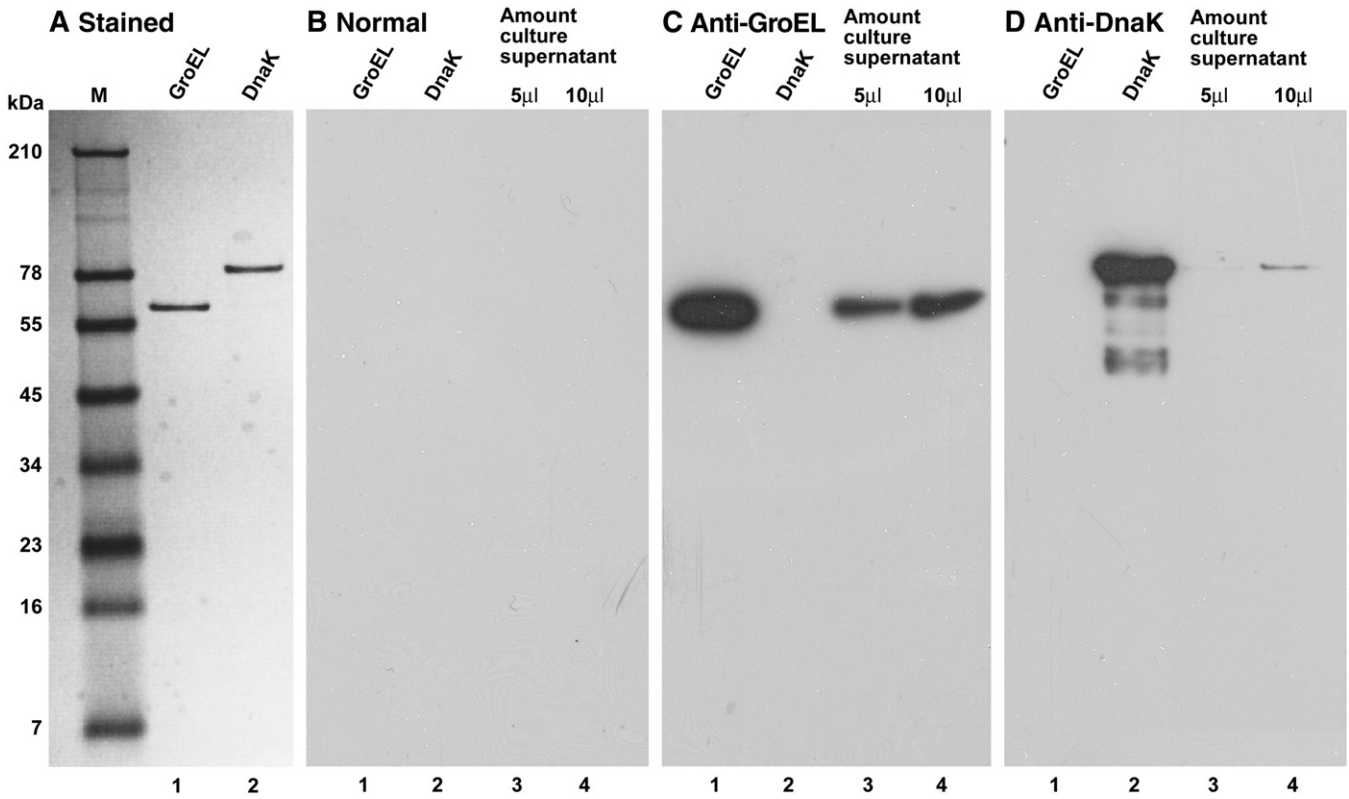

Fig. 4. A Western blot analysis for the presence of GroEL and DnaK proteins in the supernatant of a 17-h culture of *B. mallei*. Panel A is a stained gel with size markers (M) and *B. mallei* GroEL and DnaK proteins in lanes 1 and 2, respectively. Panels B to D are Western blots probed with a 1 of 5000 dilution of normal mouse serum, mouse anti-GroEL serum, or mouse anti-DnaK serum, respectively. The lanes in panels B to D contain 1) GroEL, 2) DnaK, 3) 5 μL, or 4) 10 μL of *B. mallei* cell-free culture supernatant (after 17 h of growth). All Western blots were exposed for the same length of time.

labeled with only fluorescent-label anti-GroEL protein and even less with labeled anti-DnaK protein or control serum (data not shown). We then examined the supernatant of a 17-h culture of *B. mallei* cells by Western-blot analysis to see if the hsps were excreted or released from the cell, and we found predominately GroEL protein and very little DnaK protein present (Fig. 4). We also used a *B. mallei* cell that expressed GFP as an internal protein control for premature cell lysis and found primarily GroEL protein and little DnaK protein or GFP in the supernatant after 4.5 h (data not shown) and 8.5 h of incubation (Fig. 5). However, after 24 h of incubation, when the cells had reached stationary phase, we saw DnaK protein and GFP besides GroEL protein in the supernatant, although the latter protein was still the more abundant protein. The results suggest that more GroEL protein may be released from the cell than DnaK protein during growth, and we hypothesize that this same event may occur in vivo, thereby exposing the *B. mallei* hsps to the host's immune system.

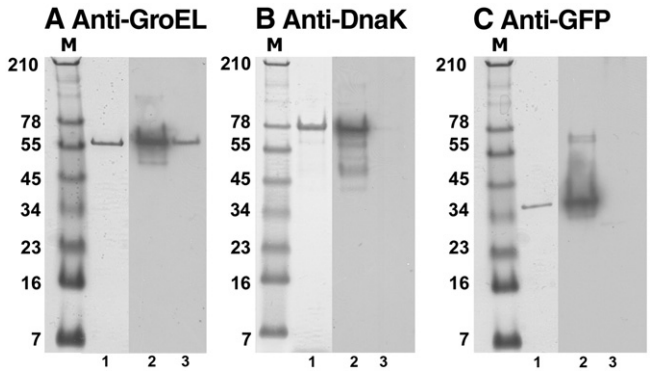

Fig. 5. A Western blot analysis for the presence of GroEL and DnaK proteins in the supernatant of an 8.5-h growing culture of a *B. mallei* strain containing GFP. In panels A to C, lane 1 is a stained gel of the GroEL, DnaK, or GFP proteins, respectively. In the same panels, lane 2 contained GroEL, DnaK, or GPF, respectively, and lane 3 contained 10 μL of a cell-free supernatant of *B. mallei* containing GFP after 8.5 h of growth at 37 C. Panel A (lanes 2 and 3) was probed with mouse anti-GroEL serum; panel B (lanes 2 and 3) was probed with mouse anti-DnaK serum; panel C (lanes 2 and 3) was probed with chicken anti-GFP serum. All antiserums were at a 1/1000 dilution, and all blots were exposed for the same length of time. M is the stained protein marker lane.

4. Discussion

Although we used PCR primers that were derived from the *B. pseudomallei groEL* gene, we cloned a *gro*EL gene from *B. mallei* that was 12 bp longer at the 3′ end than that reported for *B. pseudomallei*. This was because of an extra copy of a 12-bp direct repeat at the end of the *B. mallei groEL* gene (5′-GGCATGGGCATG-3′). The presence of

multiple direct repeats in genes from *B. mallei* was noted previously (Nierman et al., 2004). Because of the presence of a large IR downstream from the *groEL* gene, which may serve as a transcriptional termination site, the *groESL* operon appeared to consist of the *groES* and *groEL* genes. The *B. mallei dnaK* gene was clustered with the *grpE* and *dnaJ* genes, whereas in some other bacteria *dnaK*, it can be found coupled with *dnaJ* and *grp*E in a separate operon (Segal and Ron, 1996).

From the description of the medical history of the glanders patient in our study, the patient may have acquired the infection of *B. mallei* cutaneously (Srinivasan et al., 2001). Before the glanders episode, the patient had worked previously with *B. mallei*, *B. pseudomallei*, and *Burkholderia thailandensis*. The preinfection antiserum from the patient was drawn at the time of employment at this facility. However, the IgG ELISA titers against the *B. mallei* GroEL and DnaK proteins were significantly above that of healthy volunteers, and a slight reactivity against both proteins by Western blot analysis by the preinfection antiserum was obtained. Furthermore, there was a significant IgM antibody titer against the *B. mallei* whole-cell antigen when compared with the IgM antibody titer of healthy volunteers. These results suggest that the glanders patient may have been previously exposed to *B. mallei*, *B. pseudomallei*, or *B. thailandensis*, and because of the homology of the antigens among the organisms, the antibody titers in the preinfection antiserum were significantly high.

An immune response to hsps has been reported for many infectious bacteria (Zugel and Kaufmann, 1999). The major reactive hsp has been GroEL in most cases, although it has been reported that the reaction to GroEL can be variable and may even be absent in some patients. It has been also reported that the 60-kDa hsp, the GroEL homologue, is strongly immunoreactive in almost all infections by Gram-negative bacteria (Shinnick, 1991). Among these bacteria were *Brucella abortus*, *Coxiella burnetii*, *Helicobacter pylori*, *Listeria monocytogenes*, *Mycobacterium tuberculosis*, and *Yersinia enterocolitica*. Not only has a host humoral response to GroEL been detected, but also in tuberculosis and plague, there were specific populations of both CD4 αβ T-cells and CD8 αβ T-cells that recognized GroEL (Noll et al., 1994; Silva et al., 1994; Zugel and Kaufmann, 1997; Zugel et al., 1995). Depletion of γ/δ T cells in *L. monocytogenes*-infected mice resulted in a rapid increase in the number of bacteria, whereas removal of αβ T-cells from similarly infected mice caused the number of bacteria to slowly decrease over 2 weeks, when compared with infected control mice. Antigen-specific γ/δ T cells may be involved in the early defense against *Listeria* (Kimura et al., 1996), and αβ T-cells participate in the protection of the host in later stages of infection (Hiromatsu et al., 1992).

The use of biochemically purified or recombinant hsps as a vaccine has been reported to provide protection in a number of animal models of diseases. It was reported that a member of the hsp 70 family from *Histoplasma capsulatum* elicited a cellular immune response and protective immunity in vaccinated mice (Gomez et al., 1992). Subsequently, it was found that hsp 60 from *H. capsulatum* was also protective in a mouse challenge model of pulmonary histoplasmosis (Gomez et al., 1995), and that this protection was confined to a specific domain of the *H. capsulatum* hsp 60 protein (Deepe et al., 1996). A member of the hsp 60 family was also reported to be efficacious as a vaccine in a guinea pig model of Legionnaires' disease (Blander and Horwitz, 1993). Ferrero et al. (1995) reported that the GroES protein provided protective immunity against mucosal infection in mice by *H. pylori* (Ferrero et al., 1995). Intranasal inoculation of recombinant GroES protein was also used to protect mice against an intranasal challenge by *Mycobacterium avium*; however, the protection was dependent on the presence of a CpG oligodeoxynucleotide (Fattorini et al., 2002). In a *Y. enterocolitica* mouse infection model, it was found that *Y. enterocolitica* hsp 60 included immunostimulating complexes administered parenterally, induced high antibody and hsp 60-specific T-cell responses, and resulted in protective immunity to a lethal challenge (Noll and Autenrieth, 1996). If interleukin-12 was included as an adjuvant with the *Y. enterocolitica* hsp 60, this combination induced a significant *Y. enterocolitica*-specific hsp 60 T-cell response and protection against a subsequent plague challenge.

B. pseudomallei, which is closely related to *B. mallei*, causes melioidosis and is endemic in Southeast Asia. It is usually associated with wet soil, streams, rice paddies, and ponds (White, 2003). *B. mallei*, on the other hand, is closely associated with mammalian hosts. The serum of patients with melioidosis also reacted strongly to the *B. pseudomallei* GroEL protein (Woo et al., 2001). In this latter report, it was also found that there was some cross-reactivity by Western blot analysis of the GroEL protein from *B. pseudomallei* with antisera from some patients infected with *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia*. The GroEL protein from *B. pseudomallei* was reported to have 98% aa identity to the GroEL protein of *Burkholderia cepacia*, 98% identity with *Burkholderia vietnamiensis*, and 82% identity with the GroEL protein of *Bordetella pertusis*.

It was not initially clear why, in *B. mallei* infections, the GroEL protein appeared to be more immunogenic than the DnaK protein. However, it has been reported in a number of bacterial infections that GroEL (or a hsp of similar size) is associated with the outer membrane of the bacterium or is exported from the cell. In *Legionella pneumophila*, a portion of the hsp60 molecules is in the outer membrane fraction of the cell, and after heat shock, hsp60 is possibly released from the bacterium (Garduno et al., 1998a,b). It was further shown that surface-associated *L. pneumophila* hsp60 mediates host cell invasion in a HeLa cells (Garduno et al., 1998a,b). Similarly, a 66-kDa hsp from *Salmonella typhimurium*, which is related to the 65-kDa hsp of *Mycobacterium leprae*,

is secreted from the bacterium and is involved in the binding of the bacterium to intestinal mucus (Ensgraber and Loos, 1992). In 2 very different pathogens, *Clostridium difficile* (Hennequin et al., 2001) and *Haemophilus ducreyi* (Frisk et al., 1998), the GroEL protein is associated with the bacterial surface and was involved in attachment or adherence of the bacterium to the host cell. A GroEL-like protein in *Actinobacillus actinomycetemcomitans*, a pathogen associated with periodontal disease, was found in extracellular fractions of the bacterium after heat shock and was cytotoxic to transformed nontumorigenic skin keratinocytes (Goulhen et al., 1998). Subsequently, it was reported that HspA (a homologue of GroES) and HspB (a homologue of GroEL) of *H. pylori* were selectively excreted at different times during growth of the cell in culture, and that finding of these proteins in the culture supernatant was not the result of cell autolysis (Vanet and Labigne, 1998). In contrast to these results, Phadnis et al. (1996) reported that *H. pylori* undergoes spontaneous autolysis during culture, and HspB becomes associated with the cell surface. We could not completely rule out the possibility that a small fraction of *B. mallei* undergoes autolysis during growth in cell culture, thereby releasing hsps into the medium. Furthermore, in our case with *B. mallei*, there may be other reasons that DnaK protein was not as immunogenic as the GroEL protein.

One may be that DnaK may not be expressed as highly as the GroEL protein, and therefore, there may be much less protein to be presented to the host. We have found that there does appear to be much less DnaK protein than GroEL in the *B. mallei* cell pellet by Western blot analysis, which may indicate that there is much less DnaK protein expressed than GroEL protein. Another reason may be that the DnaK protein does not leak or is not excreted from the bacterium as in the case for GroEL. It could be localized within a region of the bacterium that does readily permit it to be excreted from the cell-like GroEL, where GroEL has been reported to be near the outer membrane or in the periplasmic space. Finally, there is a possibility that the DnaK protein in itself is not as immunogenic as GroEL, but we do not think that this is the case because, when purified DnaK and GroEL proteins are injected into mice, they both induce similar levels of antibodies.

Our study showed that a glanders-infected person or mice have reactive antibodies to the GroEL and DnaK proteins, depending on the experimental method of detection. This host response, however, would not differentiate between infections by *B. mallei* or *B. pseudomallei*. In a region where both microorganisms may be present, a definitive diagnosis would still require the isolation and identification of the organism from the patient or animal, and molecular and biochemical laboratory findings (Ulrich et al., 2006; White, 2003; Zysk et al., 2000). Because, at present, there is no efficacious vaccine for glanders (Amemiya et al., 2002), the *B. mallei* GroEL protein could be evaluated as part of a conjugate vaccine. It has been shown with self and foreign hsp 60 T-cell peptides with a capsular polysaccharide from *Salmonella typhi* that both humoral and T-cell immune responses are induced to the normally T-cell–independent antigen (Konen-Waisman et al., 1999). *B. mallei* is a Center for Disease Control and Prevention category B agent and has been shown to be highly infectious to both humans and animals. Because of the scarcity of human cases, there is a need of better definitive diagnostic tools and treatments for prevention.

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